UNIVERSITI PUTRA MALAYSIA

PROPHYLACTIC AND THERAPEUTIC EFFICACIES OF DIETARY ZERUMBONE SUPPLEMENTATION ON THE PATHOGENESIS OF ATHEROSCLEROSIS IN CHOLESTEROL-FED RABBITS

HEMN HASSAN OTHMAN HASSAN

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By

HEMN HASSAN OTHMAN HASSAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

November 2014

DEDICATION

This Dissertation is Dedicated to

My Beloved Wife

My Wonderful Triplets

My Lovely Parents and Siblings

All My Sincere Teachers, Lecturers and Friends

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman: Professor Noordin Mohamed Mustapha, PhD

Faculty: Veterinary Medicine

Owing to the high incidence of cholesterol-induced cardiovascular disease particularly atherosclerosis. Furthermore, difficulty in finding a relatively efficacious, non toxic, readily available and cheap naturally existing antihyperlipidaemic and antiatherogenic complementary medicine warrant the search for such an agent. On the other hand, concerning the dangerous side effects of the chemical remedies utilized as a lipid lowering agents, therefore the current study was designed to investigate the prophylactic and therapeutic efficacies of dietary zerumbone (ZER) supplementation on the formation and development of atherosclerosis in rabbits fed with high cholesterol diet. A total of 72 New Zealand white rabbits (NZW) were divided randomly on two experimental studies carried out eight weeks apart.

First experiment was designed to investigate the prophylactic efficacy of ZER in preventing early developed atheromas lesion. A total of 30 healthy rabbits were equally allotted in to five groups comprising of six animals each, namely control (CN), hypercholesterolemic diet (HCD) and ZER preventive groups (ZPI, ZPII and ZPIII). The second experimental trial is aimed at investigating the therapeutic effect of ZER in reducing the atherosclerotic lesion progression and establishment, wherein 42 healthy NZW rabbits were equally assigned to seven groups comprising of six animals each, namely control (CN), high-cholesterol diet (HCD), ZER treatment groups (ZI, ZII and ZIII), Simvastatin (SIM) group (SG) and zerumbone-simvastatin (ZER-SIM) combination group (ZSG). Rabbits in CN group were fed a standard rabbit chow, whereas those in the HCD, ZPI, ZPII, ZPIII, ZI, ZII, ZIII, SG and ZSG given a cholesterol-rich diet (1%) (1g cholesterol/100g pellet). However, rabbits in the ZPI, ZPII and ZPIII preventive groups given ZER at a dose of 8, 16 and 20 mg/kg respectively, two weeks prior to the onset of lipidemia induction and then with the course of cholesterol-rich diet as a prophylactic measure. On the other hand, those in the ZI, ZII and ZIII treatment groups were given ZER at a dose of 8, 16 and 20 mg/kg

respectively, together with SIM at a dose of 15 and 5 mg/kg/day in SG and ZSG groups, respectively as a therapeutic measure.

Rabbits were sacrificed and thoracic aortas simultaneously with the vital internal organs were collected at 10 weeks post cholesterol-feeding for the prophylactic trial and 14 weeks concerning the therapeutic trial. In regard to the second trial, rabbits received treatments for about four weeks after cessation of cholesterol-rich diet at 10^{th} weeks.

Following four weeks of supplementary treatment subsequent to high-cholesterol diet cessation at 10^{th} week of 2^{nd} experiment, ZER significantly reduce the serum lipid profile in all treated groups in a dose dependant manner as compared to non treated hypercholesterolemic animals. Sudanophilia, histopathological and ultrastructural changes show pronounced reduction in the plaque size in ZER medicated aortas. On the other hand, dietary supplementation of ZER for almost 10 weeks as a prophylactic measure indicates substantially decreasing in the lipid profile values and similarly plaque size in comparison with high-cholesterol non-supplemented rabbits.

Furthermore, the results of oxidative stress and antioxidant biomarkers evaluation indicate that ZER is a potent antioxidant in suppression the generation of free radicals in term of atherosclerosis prevention and treatment. ZER significantly reduces the value of MDA and augments the value of SOD. Zerumbone significantly reduces the incidence of inflammatory response in the process of atherosclerosis formation and development through significant suppression of proinflammatory mediators NF-κB, iNOS and COX-2, in turn reduce the inflammatory cytokines secretion TNF-α, IL-6, IL-1, and IF-$^{\gamma}$ evaluated by Western blotting and enzyme immunoassay techniques respectively.

Conversely, reduction and suppression of inflammatory mediators will contribute to minimizing the chronic inflammatory cells mainly macrophages recruitment to the lesion and foam cell formation which is evident by immunohistochemistry and fluorescent assay of RAM-11. ZER significantly reduces the expression of RAM-11 in the intimal plaque in all ZER supplemented groups in a dose dependent manner.

Moreover, ZER significantly reduces the proliferation and migration of vascular smooth muscle fibers through immunohistochemical and fluorescent detection of HHF-35 toward the intimal layer via induction of apoptosis, which is evident by down regulation of Bcl-2 and up regulation of Bax significantly evaluated by Western blotting technique. Furthermore, *in vivo* antiproliferative assay of ZER determined morphologically by TUNEL assay.

In conclusion our data indicate that, dietary supplementation of ZER at doses of 8, 16 and 20 mg/kg alone as a prophylactic measure and as a supplementary treatment with simvastatin, significantly reduces early plague formation, development, and

establishment via significant reduction in serum lipid profile together with suppression of oxidative damage, therefore alleviate atherosclerosis lesions. Simultaneously, ZER significantly suppresses inflammatory reaction within the plaque consequently prevent foam cell formation and plaque progression. Finally, ZER significantly lessens smooth muscle cells proliferation via induction of apoptosis eventually reduce the plaque size.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KEBERKESANAN DIET ZERUMBON SEBAGAI PROFILAKSIS DAN TERAPEUTIK PERKEMBANGAN ATEROSKLEROSIS PADA ARNAB

Oleh

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Berasaskan kepada insidens penyakit kardiovaskular teraruh-kolesterol terutamanya arteriosklerosis, satu kajian telah dibentuk untuk menyiasat kemujaraban profilaksis dan terapeutik pemberian zerumbon (ZER) rangsum terhadap pembentukan dan perkembangan arteriosklerosis pada arnab yang diberi rangsum tinggi kolesterol. Sebanyak 72 ekor arnab NZW dibahagikan secara rawak kepada dua kajian berjarak lapan minggu antaranya. Ujikaji pertama dibentuk untuk menyiasat kesan profilaksis kemujaraban ZER dalam mencegah pembentukan lesi awal arteroma. Sebanyak 30 ekor arnab sihat dibahagikan secara rata kepada lima kumpulan yang setiapnya mengandungi enam ekor iaitu, kawalan (CN), rangsum hiperkolesterol (HCD) dan kumpulan pencegahan ZER (ZPI, ZPII dan ZPIII).

Ujikaji kedua disasar untuk menyiasat kesan ZER dalam mengurang kemajuan serta pengukuhan lesi arteriosklerosis oleh ZER, dimana sebanyak 42 ekor arnab sihat NZW dibahagian secara rata kepada tujuh kumpulan mengadungi enam ekor arnab setiap satu, iaitu kawalan (CN), rangsum kolesterol tinggi (HCD), rawatan ZER (ZI, ZII dan ZIII), SIM (SG) dan gabungan ZER-SIM (ZSG). Arnab dalam kumpulan CN diberi makan makanan piawai arnab manakala dalam kumpulan HCD, ZPI, ZPII, ZPIII, ZI, ZII, ZIII, SG dan ZSG masing-masing diberi rangsum tinggi kolesterol (1%) (1g kolesterol/100 g makanan). Bagaimanapun, arnab dalam kumpulan pencegahan ZPI, ZPII dan ZPIII masing-masing menerima ZER pada dos 8, 16 dan 20 mg/Kg, dua minggu sebelum kejadian aruhan lipidemia dan kemudiannya dengan rawatan rangsum tinggi kolesterol sebagai langkah pencegahan.

Sebaliknya, kumpulan rawatan ZI, ZII dan ZIII menerima ZER masing-masing pada dos 8, 16 dan 20 mg/Kg bersamaan dengan SIM pada dos 15 dan 5 mg/kg/day pada kumpulan SG dan ZSG sebagai langkah terapeutik. Selepas dikorbankan, aorta toraks

bersamaan dengan organ dalam penting lain diambil pada minggu ke 10 pasca pemberian kolesterol untuk kajian profilaksis. Dalam kajian terapeutik, arnab menerima rawatan selama empat minggu selepas 10 minggu pemberian rangsum kolesterol. Sepanjang empat minggu rawatan tambahan selepas pada pemberhentian rangsum tinggi kolesterol pada minggu ke-10 di ujikaji kedua, ZER telah mengurang profil lipid serum secara keertian pada semua kumpulan rawatan dalam pola bergantung dos berbanding dengan kumpulan kolesterol tinggi tak terawat. Sudanofilia, perubahan histopatologi and ultrastruktur menunjuk pengurangan ketara saiz plak pada aorta haiwan terawat ZER.

Sebaliknya, rawatan ZER selama hampir 10 minggu sebagai langkah profilaksis menandakan pengurangan ketara nilai profil lipid dan saiz palk berbanding dengan kumpulan tak terawat. Tambahan lagi, keputusan tegasan oksidatif dan biopenanda anti-pengoksidaan menunjukkan kemanjuran ZER dalam menindas penjanaan radikal bebas berasaskan pencegahan dan rawatan arteriosklerosis. ZER juga secara keertian telah mengurang nilai MDA dan dan mengganda nilai SOD. Zerumbon telah secara keertian mengurang insidens gerakbalas inflamasi dalam proses pembentukan dan perkembangan aterosklerosis melalui penindasan keertian perantara proinflamasi seperti NF-κB, iNOS dan COX-2 yang seterusnya mengurang rembesan sitokin inflamasi seperti TNF-α, IL-6, IL-1, dan IF-$^{\gamma}$ yang dinilai melalui teknik asai penyerapan Western.

Pengurangan dan penindasan perantara inflamasi akan menyumbang kepada pengurangan sel inflamasi kronik terutamnya kemasukan makrofaj ke lesi dan pembetukan sel busa yang dibuktikan dengan imunohistokimia dan asai pendaflour RAM-11. ZER secara keertian telah mengurang penjelmaan RAM-11 pada plak intima di kesemua kumpulan pemberian tambahan ZER dengan pola bergantung dos. Tambahan lagi, ZER secara keertian mengurang pemprofileratan dan penghijrahan otot licin vaskular melalui pengesanan imunohistokimia dan pendaflour HHF-35 terhadap lapisan intima melalui paruhan apoptosis yang terbukti dengan penurunawalatur Bcl-2 dan tingkatkawalatur Bax secara keertian yang dinilai dengan teknik penyerapan Western. Tambahan lagi, anti-pemproliferatan *in vivo* oleh ZER yang dibuat secara morphologi dengan asai TUNEL.

Sebagai rumusan, data yang terhasil menunjukkan bahawa pemberian makan ZER sahaja pada dos 8, 16 and 20 mg/Kg untuk langkah profilaksis dan tambahan rawatan dengan SIM, mengurang pembetukan, perkembangan dan pengukuhan awal plak secara keertian melalui penurunan profil lipid berserta penindasan kerosakan oksidatif yang mengurang lesi arterioskeloris. Malah gabungan ZER menindas secara keertian tindakbalas inflamasi dalam plak yang akhirnya menghalang pembnetukan sel busa dan perkembangan plak. Akhir sekali, ZER secara keertian mengurang pemproliferatan otot licin melalui aruhan apoptosis yang mengecil saiz plak.

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This thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

˚C	Degree Celsius
®	Trade Mark
µg	Microgram
µl	Microlitre
µm	Micro Meter
2X	Two Fold
Å	Angstrom
AA	Arachidonic acid
ACS	Acute Coronary Syndrome
ACUC	Animal Care and Use Committee
ADP	Adenosine Di Phosphate
AGE	Advanced Glycation End Products
Alb	Albumin
ALL	Acute Lymphocytic Leukemia
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
Ang-II	Angiotensin-II
ANOVA	One-Way Analysis of Variance
apoA1	Apolipoprotein A1
AST	Aspartate Aminotransferase
Bax	Bcl_2 Associated X Protein
BDMA	Benzyl Dimethyl Amine
BHT	Butylated Hydroxytoulene
CAD	Coronary Artery Disease
CHD	Coronary Heart Diseases
Cm	Centimeter
cm^2	Square Centimetre
COX-2	Cyclooxygenase -2
CPD	Critical Point Drier
Creat	Creatinine
CRP	C-Reactive Protein
Cu Kα	Copper Anode
CVD	Cardiovascular Disease
CXCR3	Chemokine Receptor Type 3
Cyt-c	Cytochrome C
DAB	3, 3'-Diaminobenzidine
DCs	Dendritic Cells
dNTP	Deoxyribonucleotide
DPX	Mounting Media And Section Adhesive
EDTA	Ethyl Diamine Tetra Acetic Acid
EE	Ethanolic Extract
EEZZ	Ethanolic Extract of Zingiber Zerumbet Rhizome
ELISA	Enzyme Linked Immunosorbant Assay
EM	Electron Microscopy
FGF	Fibroblast Growth Factors
FITC	Fluorescein Isothiocyanate
g	Gram

GGT	Γ-Glutamyl Transferase
GSH	Glutathione
h	Hour (S)
H&E	Haematoxylin And Eosin
HCD	High-Cholesterol Diet
HDL	High-Density Lipoprotein
HMG-CoA	3-Hydroxy-3-Methyl Glutaryl Coenzyme A
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
ICAM-1	Intercellular Adhesion Molecule 1
IDL	Intermediate Density Lipoprotein
IFA	Immunoflourescent Assay
IFN- γ	Interferon- γ
IHC	Immunohistochemistry
IL-1	Interleukin-1
IL-6	Interleukin-6
iNOS	Inducible Nitric Oxide Synthase
IκBα	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Alpha
κ	Kappa
kDa	Kilo Dalton
Kg	Kilogram
KH_2PO_4	Potassium Dihydrogen Phosphate
L	Litre
LOX-1	Lectin-Type Oxidized LDL Receptor 1
MCP-1	Monocyte Chemotactic Protein 1
MDA	Malondialdehyde
MeOH	Methanol
mg	Milligram
MI	Myocardial Infarction
min	Minute
mL	Millilitre
MM	Mucus Membrane
Mm	Micromolar
mm	Millimetre
MMP	Matrix Metalloproteinases
n	Number
NaCN	Sodium Cyanide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NH_4Cl	Ammonium Chloride
nmol	Nanomole
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
OD	Optical Density
Ox-LDL	Oxidized Low Density Lipoprotein
$P < 0.05$	Probability Values of Less Than Alpha 0.05
PBS	Phosphate Buffer Saline

PDGF	Platelet-Derived Growth Factor
PGE2	Prostaglandin E2
pH	Measurement For Hydrogen Ion Concentration
PI	Propidium Iodide
PPAR-α	Peroxisome Proliferator-Activated Receptor-Alpha
RNAase	Ribonuclease Enzyme
ROS	Reactive Oxygen Species
rpm	Revolution Per Minute
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis.
Sec	Second (S)
SEM	Scanning Electron Microscope
SMC	Smooth Muscle Cells
SOD	Superoxide Dismutase
SPSS	Statistical Package For The Social Sciences
T-cell	T Lymphocyte
TEM	Transmission Electron Microscopy
TNF-α	Tissue Necrotizing Factor-Alpha
TUNEL	Tdt-Mediated Dutp Nick-End Labelling
UPM	University Putra Malaysia
v/v	Volume To Volume
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low-Density Lipoprotein
VSMC	Vascular Smooth Muscle Cells
w/v	Weight To Volume
WB	Western Blotting
WHHR	Watanabe Heritable Hyperlipidemic Rabbit
WHO	World Health Organization
ZER	Zerumbone
ZZ	Zingiber Zerumbet (L.) Smith
β-actin	Beta Actin
γ	Gamma

CHAPTER ONE

GENERAL INTRODUCTION

Atherosclerosis is a complex, chronic, proliferative and accumulative inflammatory disorder with the involvement of immune system (Ross, 1999; Galkina and Ley, 2009). It is a disease of large and medium sized arteries characterized by focal intimal thickening of the arterial wall associated with lipid deposition in the form of elevated lipid-filled plaques called atheromas (Ross *et al.*, 1984). Necrosis and fibrosis that follow the fibro-fatty plaque progression probably will result in partial or complete occlusion leading to ischemia and infarction (Ross, 1993b). It is of greatest importance of cardiovascular disease (CVD) in humans and is considered a public health issue accounting for an estimated 50% of overall deaths in western countries (O'Connor *et al.*, 2001; Stocker and Keaney, 2004).

It is generally believed that atherosclerosis is a chronic inflammatory response that is advanced by lifestyle-related disorders, such as elevated serum cholesterol particularly low density lipoprotein (LDL-cholesterol), hypertension, diabetes mellitus and cigarette smoking (Altman, 2003; Sata and Fukuda, 2011). Recently hyperhomocysteinemia (Faraci and Lentz, 2004) and infectious microorganisms such as Cytomegalovirus, herpesviruses, *Helicobacter pylori* and *Chlamydia pneumoniae* (Chiu, 1999; Ameriso *et al.*, 2001) are believed to be contributors to the initiation and development of atherosclerosis.

Numerous pathophysiological investigations in humans and animals led to the formulation of the response-to-injury hypothesis of atherosclerosis (Ross and Glomset, 1973), which principally proposed that endothelial denudation was the initial step in the process of atherogenesis (Ross and Glomset, 1976). This postulation supports the early theory suggesting that cellular responses in atherosclerosis are secondary in response to mechanical and/or toxic injuries leading to endothelial dysfunction (Von Rokitansky and Swaine, 1855). This is in contrast, to the postulated an initial and critical role of cellular pathology in the formation and development of atherosclerosis (Virchow, 1860), once more supported by Ross (Ross, 1999) and antithetical to the humoral pathology theory of the Rokitansky's school (Mayerl *et al.*, 2006).

Currently, large number of recent work emphasize that the chronic inflammatory reaction together with involvement of innate and adaptive immune response in association with the traditional risk factors play a pivotal role in the initiation and progression of atherosclerosis (Hansson and Libby, 2006; Kaperonis *et al.*, 2006; Libby, 2012). Atherogenesis comprises three fundamental stages, including intimal thickening, plaque development, and plaque destabilization-rupture (Sakata, 2012). The initial and earliest lesion of atherosclerosis the so-called fatty streaks (Ross *et al.*, 1984) is common in infant and young children (Napoli *et al.*, 1997). It is merely and simply an inflammatory lesion consisting of T-lymphocytes (T-cells), dendritic cells (DCs), and monocyte-deriving macrophages, with involvement of innate and adaptive immune systems (Hansson and Libby, 2006; Galkina and Ley, 2009).

As fatty streaks developed, in result of endothelial injury that accumulates lipid, extracellular lipid particularly low density lipid (LDL) modified by oxidative pathway and engulfed by macrophages to form immobile foam cells (Stary *et al.*, 1994). Cytokines, growth factors, adhesion molecules and chemotactic proteins are released by chronic inflammatory cells and denudated endothelial cells result in monocytes recruitment, extracellular matrix production and smooth muscle cells (SMCs) proliferation-migration and transformation to foam cells in the intima (Lusis, 2000).

Foam cells originated from transformed SMCs that are subjected to death by apoptosis (Okura *et al.*, 2000). Progression of atherosclerosis from early to advance lesions will initiate with the generation of lipid-rich core in the deep layer of thickened intima derived from dead foam cells containing necrotic tissues and free cholesterol crystals which is called atheroma, that wrapped by thick fibrous cap (Ross, 1993a). Continous atheroma build up result in arterial luminal narrowing particularly in the coronary artery, and it is considered a primary cause of stable angina pectoris (Sakata, 2012).

Persistent recruitment of inflammatory cells into the lesion particularly macrophages render the fibrous cap thin and make the plaque weak in architecture (Lendon *et al.*, 1991), therefore, more vulnerable to rupture in response to the physical forces of blood flowing causes ulceration, hemorrhage, and thrombus formation, which result in sudden death from myocardial infarction and stroke (Ross, 1999). Coronary artery disease (CAD) arising from atherosclerosis is a leading cause of mortality worldwide. Currently available therapeutics against atherosclerosis is basically limited to alleviating the traditional risk factors such as hyperlipidemia and hypertension or controlling the thrombotic complications (Weber and Noels, 2011).

Treatment with statin drugs in patients with CAD shows a significant reduction in risk factors that is correlates to LDL-cholesterol, thereby limiting plaque development and reduces possibly of plaque rupture throughout its pleiotropic anti-inflammatory and antihypercholesterolaemic effects, endothelial dysfunction improvement, and reducing thrombogenicity (Ray and Cannon, 2005). Furthermore, monitoring high blood pressure with antihypertensive beta-blockers contributes to lower mortality from myocardial infarction and stabilized atheroprogression (Sipahi *et al.*, 2007). More recently, medication with artificial peptide-based high density lipoprotein (HDL)-like apolipoprotein as an additive in statin-treated patients exert a profound anti-inflammatory and lipid lowering effects (Navab *et al.*, 2010).

On the other hand, concerning the dangerous side effects of these chemical remedies such as high doses of statins in patients with high serum cholesterol have been found to cause striated muscle damage (Antons *et al.*, 2006), and increase the risk of rhabdomyolysis associated with neuropathy (Fadini *et al.*, 2010). In addition to the complicated nature and pathogenesis of atherosclerosis, involving oxidative stress damage, endothelial dysfunction (Victor *et al.*, 2009), elevated level of LDL-cholesterol and chronic inflammatory reaction (Libby *et al.*, 2002; Kaperonis *et al.*, 2006).

As a result, seeking a multifaceted natural product-based complementary and alternative herbal medicine, which is readily available, effective, and addressed all major risk factors with no toxic effects, was the best choice in providing an ideal remedy in the prevention and treatment of atherosclerosis (Zeng *et al.*, 2012).

The usage, interest, and self-administration of herbal medicine are widespread and most popular alternative therapy among patients under CVDs pharmacotherapy (Izzo *et al.*, 2005). Using natural compounds as a supplementary healthy diet is not a substitute for regular medical care, however, it is considered as a complementary and alternative medicine (Brown *et al.*, 2007). Medical herbs and plant foods such as fruits, vegetables, and spices contain many biologically active phytochemical compounds that have various health promoting effects (Lampe, 1999).

Zingiber zerumbet (L.) Smith belonging to Zingiberaceae, is an edible ginger, originating from South-East Asia has been cultivated thousands of years as a spice-food additive and for medical purposes (Vimala *et al.*, 1999). Whereas, approximately 161 species from 18 genera of this family are found in Peninsular Malaysia (Ruslay *et al.*, 2007). The extracts of *Zingiber zerumbet* rhizomes, have been used as a traditional medicine to treat various types of inflammatory mediated human ailments as a potent inflammatory suppresser contribute to down regulate the proinflammatory mediators such as prostaglandin E2 (PGE2) (Chien *et al.*, 2008).

Recently, the rhizome's extract have been extensively studied in multiple investigations for its effectiveness in a broad range of biological activities include antinociceptive (Sulaiman *et al.*, 2009), anti-inflammatory (Zakaria *et al.*, 2010), antioxidant (Yob *et al.*, 2011), antimicrobial (Habsah *et al.*, 2000), antifungal (Jantan *et al.*, 2003), antitumor (Kirana *et al.*, 2003) and antiplatelet aggregation (Jantan *et al.*, 2008). More recently, ethanolic extract of *Zingiber zerumbet* showed antihypercholesterolaemic property in rats fed a high-fat diet (Chang *et al.*, 2012).

Of all bioactive compound(s) isolated and identified from various extracts of *Zingiber zerumbet* rhizomes, zerumbone has been studied extensively due to its broad-spectrum biomedical properties. Zerumbone (ZER) is a crystalline, monocyclic, sesquiterpene, phytochemical substance that was first isolated as a major compound in 1960 from the essential volatile oil of rhizomes of *Zingiber zerumbet* (L.) smith (Kitayama *et al.*, 2003). It predominantly can be isolated from both leaves and rhizomes of the plant at approximately 36.98% and 46.83%, respectively (Bhuiyan *et al.*, 2008).

Numerous biological and therapeutical activities of ZER includes anticancer and antioxidant (Murakami and Ohigashi, 2006), anti-inflammatory (Somchit *et al.*, 2012), antinociceptive (Zakaria *et al.*, 2010), antimicrobial (Kader *et al.*, 2011), hepatoprotective (Fakurazi *et al.*, 2009), antiproliferative and apoptosis inducing agent (Sakinah *et al.*, 2007b) and immunomodulatory activities (Keong *et al.*, 2010) in a dose dependant manner. In addition, ZER has been demonstrated to attenuate inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression via modulation of nuclear factor kappa-B cells (NF-κB) activation (Takada *et al.*, 2005; Murakami and Ohigashi, 2007), beside its potent anti-inflammatory (Sulaiman *et al.*, 2010) and antioxidant (Murakami *et al.*, 2003) efficacy.

Additionally, antiproliferative and apoptotic inducing effects of ZER been approved against variety of organ carcinogenesis via down and up regulation of Bcl-2 and Bax, respectively (Murakami *et al.*, 2002; Takada *et al.*, 2005; Sakinah *et al.*, 2007a). Moreover, ZER evidently approved to suppress some cytokines that play a major role in the process of atherogenesis such as interleukin-6 (IL-6) (Abdelwahab *et al.*, 2012) and tissue necrotizing factor-alpha (TNF-α) (Chen *et al.*, 2011).

Depend upon the mentioned phytothearaputic effects of ZER against proliferative and inflammatory diseases via suppression of proinflammatory mediators and cytokines (Sulaiman *et al.*, 2010). In addition to its active antioxidant property in suppressing free radicals generation and its potent antiproliferative activity via upregulaiton of proapoptotic genes (Murakami *et al.*, 2002). Furthermore, to date, no study has addressed the effect of ZER on serum lipid profile in relation to atherosclerosis progression in rabbits. Therefore, the general objective of this study is to investigate the prophylactic and therapeutic efficacies of dietary ZER supplementation on the development of atherosclerosis in rabbits fed with high cholesterol diet.

Problem Statement

Difficulty in finding a relatively efficacious, non-toxic, readily available, cheap, and naturally existing antiatherogenic and antihyperlipidaemic agent warrants the search for such an agent.

Hypothesis

Null Hypothesis

Zerumbone supplementation shows no lipid lowering effect and not an antiatherogenic agent, thus will not prevent and reduce the early development of atherosclerotic lesions in hypercholesterolemic rabbits.

Alternative or Research Hypothesis

Zerumbone will prevent and reduce the development of atherosclerotic plaques induced by high cholesterol diet via suppression and down regulation of proinflammatory mediators and cytokines thus inflammatory reaction. As well as, inducing apoptosis and reducing smooth muscle cell proliferation-migration in turn plaque propagation. Finally yet importantly, suppressing free radicals production hence, minimizing oxidative stress damage, and lowering lipid profile subsequently alleviate plaque development.

Aim and objectives

The main aim of the study is to evaluate the prophylactic and therapeutic efficiencies of ZER supplementation on the formation, development, and establishment of early atherosclerosis in rabbits fed with high-cholesterol diet with the following objectives:

1) To evaluate the antihypercholesterolaemic effect of dietary ZER supplementation on the initiation and propagation of atherosclerosis in cholesterol-fed rabbits.

2) To estimate the antioxidant efficacy of dietary ZER supplementation on the formation and development of atherosclerosis in hypercholesterolemic rabbits.

3) To assess the anti-inflammatory effect of ZER supplementation on the initiation and development of atherosclerosis in rabbits fed high-cholesterol diet.

4) To estimate the antiproliferative and apoptosis inducing effect of ZER supplementation on the formation and progression of atherosclerosis in rabbits on high-cholesterol diet.

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